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(54) **A regulatory factor involved in expression of nitrilase gene, and its gene**

(57) The invention relates to a regulatory factor substantially containing an amino acid sequence represented by SEQ ID NO:1 and having the action of activating a nitrilase gene promoter, a regulatory factor gene containing DNA coding substantially for said regulatory factor, a recombinant plasmid containing said regulatory factor gene, a nitrilase gene containing a promoter region and a DNA region capable of replicating in a micro-

organism belonging to the genus *Rhodococcus*, and a transformant transformed with said recombinant plasmid.

According to the present invention, there are provided a regulatory factor gene containing DNA coding substantially for a regulatory factor having the action of activating a nitrilase gene promoter, a recombinant plasmid containing said regulatory factor gene, and a transformant transformed with said plasmid.

**EP 0 759 474 A2**

**Description****FIELD OF THE INVENTION**

5 The present invention relates to a regulatory factor involved in expression of a nitrilase gene as well as to DNA coding for said regulatory factor. In particular, the present invention relates to a regulatory factor derived from *Rhodococcus rhodochrous* J1 and having the action of activating a nitrilase gene promoter, a recombinant plasmid containing DNA coding for said regulatory factor, a nitrilase gene promoter and a nitrilase gene, and a transformant transformed with said recombinant plasmid.

**BACKGROUND OF THE INVENTION**

Organic acids can be formed from their corresponding nitriles under mild conditions by use of microorganisms or microorganism-derived enzymes (e.g. nitrilase) as catalysts (see Japanese Unexamined Patent Publication (hereinafter referred to as "Kokai") Nos. Sho 58-201992, Sho 61-40795, and US 5,283,193 (Hei 2-84198 and Hei 3-251192).

As compared with conventional processes, the catalytic ability of microorganisms to hydrolyze nitriles is expected to be drastically improved by use of recombinants carrying a cloned nitrilase gene because they can be engineered to contain multiple copies of the gene.

10 In order to prepare a microorganism as a catalyst having higher catalytic activity, the present inventors cloned a nitrilase gene from *Rhodococcus rhodochrous* J1 and constructed a plasmid by inserting the gene to a region downstream of *E. coli* lactose promoter (J. Biol. Chem. 267, 20746-20751 (1992)). *E. coli* into which said plasmid had been introduced exhibited higher nitrilase activity when cultured in the presence of IPTG (isopropyl- $\beta$ -D-thiogalactoside).

To further improve a bacterial catalyst for utility value, a recombinant was prepared from a microorganism belonging to the genus *Rhodococcus* with superior functions by integrating a nitrilase gene region into a *Rhodococcus-E. coli* hybrid plasmid vector (see Kokai Nos. Hei 5-64589 (=EP 0502476A) and 5-68566 (=EP 0502476A)) and introducing it into a microorganism belonging to the genus *Rhodococcus*.

However, no nitrilase activity was attained by the transformant into which the nitrilase gene region had merely been introduced.

Hence, it was desired to develop a transformant of the genus *Rhodococcus* by which nitrilase activity can be 20 obtained.

**SUMMARY OF THE INVENTION**

The object of the present invention is to provide a regulatory factor derived from *Rhodococcus rhodochrous* J1 and having the action of activating a nitrilase gene promoter, a recombinant plasmid containing DNA coding for said regulatory factor, a nitrilase gene promoter and a nitrilase gene, and a transformant transformed with said recombinant plasmid.

The present inventors speculated that the reason the gene is not expressed by the transformant derived from the genus *Rhodococcus* is that the promoter for the nitrilase gene fails to function because the transformant does not carry a gene coding for a regulatory factor necessary for functioning of the promoter. Hence, they thought that a gene coding for such regulatory factor is present somewhere in chromosomal DNA from the J1 strain and found the gene located downstream of the nitrilase structural gene. As a result of preparation of a transformant belonging to the genus *Rhodococcus* carrying this gene, the transformant could successfully express nitrilase with high activity.

That is, the present invention is a regulatory factor substantially containing an amino acid sequence represented by SEQ ID NO:1 and having the action of activating a nitrilase gene promoter. This action of the regulatory factor is enhanced by the presence of nitriles such as isovaleronitrile.

Further, the present invention is a regulatory factor gene containing DNA coding substantially for an amino acid sequence represented by SEQ ID NO:1.

Further, the present invention is a recombinant plasmid containing said regulatory factor gene, a nitrilase gene containing a promoter region, and a DNA region capable of replicating in a microorganism belonging to the genus *Rhodococcus*. As the DNA region capable of replicating in a microorganism belonging to the genus *Rhodococcus*, mention may be made of a member selected from a group consisting of plasmids pRC001, pRC002, pRC003 and pRC004.

The terms "substantially containing" and "coding substantially for" an amino acid sequence are intended to indicate that insofar as the peptide of the amino acid sequence maintains the function of activating the nitrilase gene promoter, the amino acid sequence may have deletion, replacement, addition etc. of amino acids. Hence, an amino acid sequence represented by SEQ ID NO:1 but with deletion etc. of the 1st amino acid methionine (Met) is understood as an intended polypeptide with an alternation of amino acid. Further, the present DNA coding for such polypeptide, which is repre-

sented by a nucleotide sequence represented by SEQ ID NO:2, includes degenerated isomers coding for the same polypeptide with different degenerated codons.

Further, the present invention is a transformant transformed with said recombinant plasmid.

## 5 BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a restriction enzyme map in the vicinity of the nitrilase gene from the J1 strain, plasmids constructed in the present invention, and nitrilase activities of transformants carrying these plasmids.

## 10 DETAILED DESCRIPTION OF THE INVENTION

The gene of the present invention contains a region coding for a regulatory factor having the action of activating a nitrilase gene promoter (referred to hereinafter as "regulatory factor") and it is prepared in the following steps.

### 15 (1) Construction of a plasmid containing a nitrilase gene and a gene coding for the regulatory factor:

The nitrilase gene derived from *Rhodococcus rhodochrous* J1 (referred to hereinafter as "the J1 strain") is known (J. Biol. Chem. 267, 20746-20751 (1992)) and obtained as plasmid pNJ10 having this gene inserted into vector pUC19. The nitrilase gene can be prepared by digesting this plasmid pNJ10 with suitable restriction enzymes such as PstI etc.

20 The nitrilase gene and the gene coding for the regulatory factor are included in the gene fragment thus prepared.

A plasmid for use in ligation of this gene fragment containing the nitrilase gene includes e.g. hybrid plasmids such as pK1, pK2, pK3 and pK4.

The plasmid pK1 is a hybrid plasmid between plasmid vector pHSG299 for *E. coli* and plasmid pRC001 which is a DNA region capable of replicating in a microorganism belonging to the genus *Rhodococcus*. Hybrid plasmids between plasmid vector pHSG299 and plasmids pRC002, pRC003 and pRC004 are designated pK2, pK3 and pK4 respectively

25 (see Kokai No. Hei 5-68566). In the present invention, the hybrid vector pK4 will be exemplified.

Then, the above fragment containing the nitrilase gene and the gene coding for the regulatory factor is ligated to the aforementioned hybrid plasmid. Ligation may be carried out in any known method. For example, a commercially available ligation kit (Takara ligation kit available from Takara Shuzo Co., Ltd.) can be used in ligation.

30 The plasmid DNA thus obtained contains the nitrilase gene and the gene coding for the regulatory factor, but where the gene coding for the regulatory factor is located in this plasmid DNA is not certain.

Hence, this plasmid DNA is treated with various restriction enzymes to prepare DNA fragments varying in size which are transformed into microorganisms belonging to the genus *Rhodococcus*. The location of the gene coding for the regulatory factor can be determined on the basis of the sizes of DNA fragments permitting the transformants to express nitrilase.

35 The gene fragment containing the nitrilase gene and the gene coding for the regulatory factor can be ligated to said plasmid in the correct direction or reverse direction in 50 % probability.

The J1 strain has been deposited as FERM BP-1478 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan. The plasmid pNJ10 containing the nitrilase gene and the gene coding for the regulatory factor has been deposited as recombinant *E. coli* JM109/pNJ10 (FERM BP-5548) and the hybrid plasmid vector pK4 has been deposited as recombinant *R. rhodochrous* ATCC 12674/pK4 (FERM BP-3731) containing the same.

### 45 (2) Preparation of a transformant of the genus *Rhodococcus* and measurement of nitrilase activity

The plasmid DNA prepared in (1) above is introduced to a microorganism belonging to the genus *Rhodococcus* e.g. *Rhodococcus rhodochrous* (ATCC12674). That is, *Rhodococcus rhodochrous* (ATCC12674) at the logarithmic growth phase is harvested by centrifugation, washed and mixed with the plasmid DNA. Then, a known method e.g. electroporation etc. is used to introduce the plasmid DNA into the microorganism. The screening of the transformants can be carried out by culturing them in kanamycin-containing medium, and the target transformant can be obtained as a kanamycin-resistant colony.

50 Then, the resulting transformant is cultured in conventional medium containing polypeptone, yeast extract, malt extract etc. to give a bacteria suspension, then benzonitrile is added to the suspension as a substrate for nitrilase, and benzoic acid formed is determined in HPLC etc. If the nitrilase gene is successfully expressed, benzonitrile is converted into benzoic acid by the action of the nitrilase produced. The presence or absence of the expression of the regulatory factor gene (the activation of the nitrilase gene promoter) can be confirmed in terms of the expression of the nitrilase gene by examining the presence or absence of the formation of benzoic acid.

Isovaleronitrile, known as a good inducer for the J1 strain nitrilase, can further be added to attain higher nitrilase

activity.

### (3) Nucleotide sequencing

The gene is obtained from the transformant whose activity was observed in (2) above, and its nucleotide sequence is determined. Nucleotide sequencing can be effected using any of the known methods, e.g. the chain termination method (Sanger F., Science, 214, 1205-1210 (1980)).

The nucleotide sequence (1286 bp) between the BsmI site of the nitrilase gene and the PvuI site downstream of the gene has already been revealed in the gene thus obtained (J. Biol. Chem. 267, 20746-20751 (1992)), and thus the nucleotide sequence from the PvuI site to the EcoRI site further downstream of the gene is determined. The amino acid sequence of the regulatory factor of the present invention can be deduced from the determined nucleotide sequence.

### EFFECT OF THE INVENTION

According to the present invention, there are provided a regulatory factor having the action of activating a nitrilase gene promoter, a gene containing DNA coding substantially for said regulatory factor, a recombinant plasmid containing said gene, and a transformant transformed with said plasmid.

Nitrilase is produced by said transformant. The regulatory gene and the nitrilase gene promoter can also be used in highly expressing other kinds of protein.

### PREFERRED EMBODIMENTS OF THE INVENTION

Hereinafter, the present invention will be illustrated in detail by reference to the following example, which however is not intended to limit the scope of the invention.

The following abbreviations are used in the example. TE: 10 mM Tris-HCl (pH 7.8)-1 mM EDTA (pH 8.0). MY medium: 1 % polypeptone, 0.3 % yeast extract, 0.3 % malt extract, 1 % glucose.

#### Example 1

##### (1) Construction of a plasmid containing a nitrilase gene and a regulatory gene

From a plasmid containing a nitrilase gene fragment from the J1 strain, said gene fragment (5.4 kb PstI fragment) was excised and inserted into the PstI site of the hybrid plasmid vector pK4 whereby a recombinant plasmid was constructed. Other plasmids were also prepared by inserting a partial region from the 5.4 kb PstI fragment into pK4. The detailed procedures are as follows.

First, the 5.4 kb PstI fragment was excised from plasmid pNJ10 obtained by inserting the 5.4 kb PstI fragment containing the nitrilase gene from the J1 strain into vector pUC19 (J. Biol. Chem. 267, 20746-20751 (1992)).

Separately, PstI-cleaved pK4 was prepared in the following manner. Three microliters of reaction buffer (10 ×), 2 μl of restriction enzyme PstI and 15 μl of sterilized water were added to 10 μl of pK4 and the mixture was allowed to react 37°C for 2 hours. After an equal amount of TE-saturated phenol was added to the reaction solution, the solution was stirred and separated into upper (aqueous) and lower layers by centrifugation. The upper layer was extracted again with TE-saturated phenol in the same manner and further extracted twice with an equal amount of chloroform in the same manner. Three microliters of 3 M sodium acetate and 90 μl of ethanol were added to the upper layer, and the sample was allowed to stand at -80°C for 30 minutes, centrifuged, dried and dissolved in TE.

Then, 3 μl of the DNA fragment fraction containing the 5.4 kb PstI fragment was allowed to react with 1 μl of the above PstI-cleaved pK4 overnight at 4 °C using Takara ligation kit (available from Takara Shuzo Co., Ltd.), whereby the 5.4 kb PstI fragment was inserted into pK4. After the reaction was finished, the reaction solution was transformed into E. coli JM109. From the transformants thus obtained, plasmids pYHJ10 and pYHJ10R were prepared by the method of Birnboim and Doly (Nucleic Acids Res. 7, 1513 (1979)). The plasmid pYJ10R contained the insert in the reverse direction.

Plasmids pYHJ20 to pYHJ50 containing a partial region from the 5.4 kb PstI fragment and plasmids pYHJ20R to pYHJ50R containing the corresponding insert in the reverse direction were constructed in the same manner.

The restriction enzyme and means used were as follows: pYHJ20 and pYHJ20R:

Pst-EcoT22I fragment was inserted into the PstI site of pK4.

pYHJ30 and pYHJ30R:

Plasmid pNJ10 was digested with PstI and then blunt-ended with T4 DNA polymerase. It was then cleaved with NaeI, and the fragment was separated and recovered by electrophoresis (0.7 % agarose). Separately, pK4 was cleaved with PstI and then blunt-ended with T4 DNA polymerase, and the above fragment was inserted into the resulting blunt end.

pYHJ40 and pYHJ40R:

Plasmid pNJ10 was cleaved with NheI and EcoT22I and blunt-ended with T4DNA polymerase. The fragment was separated and recovered by electrophoresis (0.7 % agarose). Separately, pK4 was cleaved with PstI and then blunt-ended with T4 DNA polymerase, and the above fragment was inserted into the resulting blunt end.

pYHJ50 and pYHJ50R:

Plasmid pNJ10 was cleaved with NheI and blunt-ended with T4 DNA polymerase. It was then cleaved with NaeI, and the fragment was separated and recovered by electrophoresis (0.7 % agarose). Separately, pK4 was cleaved with PstI and then blunt-ended with T4 DNA polymerase, and the above fragment was inserted into the resulting blunt end.

## (2) Preparation of a transformant of the genus *Rhodococcus* and determination of nitrilase activity

The respective plasmids thus obtained were introduced into *Rhodococcus rhodochrous* ATCC12674 and the resulting transformants were examined for their nitrilase activity.

The introduction of the plasmids into the microorganism was carried out as follows: *Rhodococcus rhodochrous* ATCC12674 at the logarithmic growth phase was harvested by centrifugation, washed 3 times with ice-cold sterilized water and suspended in 15 % PEG 6000 (polyethylene glycol 6000) to a final concentration of at least  $10^9$  cells/ml. One microgram of plasmid DNA was mixed with 100  $\mu$ l of the bacterial suspension and the mixture was cooled on ice. This mixture of DNA and bacteria was introduced into a gene pulser chamber, cooled on ice and pulsed with an electrostatic capacity of 25  $\mu$ F, a resistance of 400  $\Omega$  and a voltage of 20 kV/cm. The bacterial suspension thus treated was placed on ice for 10 minutes and heated at 37°C for 5 minutes. One milliliter of MY medium was added to the suspension which was then shaken at 25 °C for 3 hours. The bacterial suspension was plated on an MY agar plate containing 50  $\mu$ g/ml kanamycin and incubated at 28°C for 2 days. The colony grown on the plate was plated on another MY agar plate containing kanamycin, and their resistance to kanamycin was ascertained by their growth on the plate.

The transformant of the genus *Rhodococcus* thus obtained was incubated at 28 °C for 2 days in a medium consisting of 10 g glycerol, 5 g polypeptone, 3 g yeast extract, 3 g malt extract, 1 g  $\text{KH}_2\text{PO}_4$ , 1 g  $\text{K}_2\text{HPO}_4$ , 0.01 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (pH 7.0)/1 L medium. After isovaleronitrile (0.5 g/L and 1 g/L) known as a good inducer for the J1 nitrilase was added to it, the transformant was incubated in the same manner.

The bacterial cells were harvested by centrifugation, and the pellet was washed with 10 mM potassium phosphate buffer (pH 7.5) and then suspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol.

Their nitrilase activity was determined as follows.

The bacterial suspension (0.25 ml) was diluted with a suitable amount of water, and 0.25 ml of 0.1 mM potassium phosphate buffer (pH 7.0) and 0.5 ml of 12 mM benzonitrile were added to it. They were allowed to react at 20°C for 10 minutes and then 0.1 ml of 1 N HCl was added to stop the reaction. The benzoic acid formed by the enzymatic reaction was analyzed by HPLC.

The results are shown in FIG. 1. In FIG. 1, the two arrows (large arrows) along the DNA fragment from the J1 strain indicate the location and direction of the nitrilase gene and the regulatory gene found in the present invention. The region assigned to each plasmid is the J1-derived DNA region inserted into vector pK4, and the arrows (small arrows) indicate the location and direction of the lac promoter on vector pK4.

The level of nitrilase activity in FIG. 1 is shown as "+" (weak) to "+++" (strongest). The symbol "-" indicates that no activity could be detected.

Nitrilase activity could be detected for the transformants carrying plasmids pYHJ10 and pYHJ10R, and particularly high activity was observed where isovaleronitrile was added. Similar results were obtained for plasmids lacking in a region 1.4 kb or further downstream from the nitrilase gene (pYHJ20 and pYHJ20R) and plasmids lacking in upstream and downstream regions of the nitrilase gene (pYHJ40 and pYHJ40R). However, no activity was detected for plasmids lacking in a region about 0.5 kb or further downstream from the nitrilase gene (pYHJ30 and pYHJ30R).

From the foregoing, it became evident that the gene for the regulatory gene is located in a downstream region very close to the nitrilase structural gene (see FIG. 1).

To determine which plasmid conferred the highest activity on the transformant, the transformants carrying pYHJ10R

to pYHJ50R were examined for their nitrilase activity in the same manner as above.

The results are shown in Table 1. In Table 1, the transformant carrying pYHJ20R exhibited the highest activity.

Table 1

Nitrilase Activities of Transformants			
transformants	isovaleronitrile	protein conc. (mg/ml)	specific activity (U/mg)
ATCC12674/pK4	-	3.48	N.D.
	+	2.03	N.D.
ATCC12674/pYHJ10R	-	2.52	0.003
	+	4.26	0.193
ATCC12674/pYHJ20R	-	4.46	0.019
	+	2.27	0.537
ATCC12674/pYHJ30R	-	4.46	N.D.
	+	8.52	N.D.
ATCC12674/pYHJ40R	-	5.04	0.002
	+	4.83	0.297
ATCC12674/pYHJ50R	-	4.61	N.D.
	+	4.49	N.D.
N.D.: not detected.			

### (3) Nucleotide sequencing

Because the nucleotide sequence downstream from the nitrilase gene to the PvuI site was revealed (J. Biol. Chem. 267, 20746-20751 (1992)), about 1.4 kb nucleotide sequence from the PvuI site to the EcoRI site was determined. Nucleotide sequencing was carried out according to the chain termination method using Tth DNA polymerase (Sanger F., Science, 214, 1205-1210 (1980)).

The result is shown in SEQ ID NO:3. The analysis of the nucleotide sequence of SEQ. ID NO:3 revealed the presence of a single long open reading frame coding for the amino acid sequence of SEQ ID NO:1. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:2. The amino acid sequence deduced from the nucleotide sequence of SEQ ID NO:2 is shown in SEQ ID NO:1.

SEQUENCE LISTING

5 SEQ ID NO: 1:

LENGTH: 319 amino acids

TYPE: amino acid

10 TOPOLOGY: linear

MOLECULE TYPE: protein

15 SOURCE

ORGANISM: Rhodococcus rhodochrous

STRAIN: J1

20 SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Asn Thr Phe Phe Ser Ser Asp Gln Val Ser Ala Pro Asp Arg

25 1 5 10 15

Val Ala Leu Trp His Asp Val Ile Cys Arg Ser Tyr Val Pro Leu

30 20 25 30

Asn Ile Thr Leu Thr Ser Glu Gln Pro Phe Ile Gly Thr Val Ser

35 35 40 45

Thr Gly Asn Leu Gly Thr Val Arg Ile Ala Thr Ser Ser Ser Leu

40 50 55 60

Pro Gln Gln Ile Thr Arg Thr Arg Arg Leu Ile Arg Gln Asp Glu

45 65 70 75

Arg Glu Tyr Leu Met Val Gly Val Gln Ser Ala Gly His Ala Leu

50 80 85 90

Val Gln Gln His Gly Arg Thr Ala Arg Val Gly Arg Gly Gly Leu

55 95 100 105

Val Phe Trp Asp Thr Arg His Pro Tyr Asp Ile Leu Phe Pro Thr

60 110 115 120

Asp Trp Arg Met Ser Val Phe Gln Phe Pro Arg Tyr Ser Phe Gly

	125	130	135
5	Phe Thr Glu Asp Phe Ile Gly Arg Met Thr Ala Val Asn Val Gly		
	140	145	150
	Gly Asp Arg Gly Ile Gly Arg Val Val Ser Ser Phe Met Thr Ser		
10	155	160	165
	Ile Asn Asp Ala Thr Asp Ala Gly Asp Leu Ala Glu Val Ala Ser		
	170	175	180
15	Leu His Asn Ser Ala Val Asp Leu Leu Ser Ala Ala Ile Arg Thr		
	185	190	195
20	Glu Leu Ala Asp Gln Ala Ala Ala Ser Asp Gly Leu Leu Glu Cys		
	200	205	210
	Val Leu Ala Tyr Ile Arg Gln Asn Leu Ala Asp Pro Asn Leu Cys		
25	215	220	225
	Ala Ser Gln Ile Ala Ala Glu His Asn Val Ser Val Arg Thr Leu		
	230	235	240
30	His Arg Leu Phe Ser Ala Thr Gly Gln Gly Val Ala Glu His Ile		
	245	250	255
35	Arg Asn Leu Arg Leu Glu Arg Ile Lys Thr Glu Leu Ala Asp Pro		
	260	265	270
	Thr Ser Arg Arg Tyr Thr Ile Ser Ala Leu Ala Arg Lys Trp Gly		
40	275	280	285
	Phe Leu Asp Pro Ser Thr Phe Ser Arg Ala Phe Lys Asp Ala Tyr		
	290	295	300
45	Gly Ile Thr Ala Arg Glu Trp Ala Ala Ser Ala Ser Ala Ser Pro		
	305	310	315
50	Thr Glu Val Ser		319

55 SEQ ID NO: 2:



LENGTH: 960 base pairs

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

SOURCE

ORGANISM: Rhodococcus rhodochrous

STRAIN: J1

SEQUENCE DESCRIPTION: SEQ ID NO: 2:

20	ATG AAC ACT TTC TTC TCC TCA GAC CAG GTC TCG GCG CCC GAT CGC	45
	GTC GCG CTC TGG CAC GAT GTC ATC TGC CGT AGC TAT GTC CCG CTC	90
	AAC ATC ACC CTC ACG AGC GAG CAA CCC TTC ATC GGT ACG GTC TCG	135
25	ACG GGC AAC TTG GGC ACG GTA CGT ATC GCG ACG TCC TCG TCA CTG	180
	CCC CAA CAG ATC ACC CGC ACT CGT CGC TTG ATC AGG CAG GAC GAG	225
	CGT GAG TAC CTC ATG GTT GGG GTG CAG TCC GCC GGC CAT GCA CTC	270
30	GTG CAG CAG CAC GGC AGA ACT GCA CGA GTC GGT CGC GGT GGA CTG	315
	GTC TTC TGG GAC ACC CGC CAT CCC TAC GAC ATC CTC TTC CCG ACA	360
	GAC TGG AGG ATG AGC GTA TTC CAG TTC CCG CGA TAC TCT TTC GGC	405
35	TTC ACC GAA GAC TTC ATC GGC AGG ATG ACC GCG GTG AAC GTC GGG	450
	GGC GAT CGC GGT ATC GGC CGA GTG GTT TCA TCC TTC ATG ACA AGC	495
40	ATC AAC GAT GCG ACC GAC GCA GGA GAC TTG GCG GAG GTA GCT TCA	540
	CTC CAC AAC AGT GCT GTC GAT CTT CTG TCA GCG GCG ATA CGG ACC	585
	GAG CTT GCC GAT CAA GCC GCC GCC TCC GAC GGC CTA CTC GAG TGT	630
45	GTG CTC GCG TAT ATC CGA CAG AAC CTG GCC GAC CCG AAC CTG TGT	675
	GCC TCA CAG ATC GCG GCG GAA CAC AAC GTC TCT GTG CGG ACC CTC	720
	CAC CGA CTG TTC TCG GCC ACG GGA CAG GGC GTG GCC GAA CAC ATC	765
50	CGT AAC CTC CGA CTC GAG CGC ATC AAG ACT GAG CTG GCA GAC CCA	810
	ACG AGC CGG CGA TAT ACG ATC AGC GCT TTG GCG AGA AAA TGG GGG	855
55	TTC CTC GAT CCC TCA ACG TTC TCA CGC GCG TTC AAA GAC GCC TAC	900

GGC ATC ACT GCC CGA GAG TGG GCG GCT TCT GCA TCA GCA TCA CCG 945  
ACG GAG GTT TCG TAG 960

SEQ ID NO: 3:

LENGTH: 1390 base pairs

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

SOURCE

ORGANISM: *Rhodococcus rhodochrous*

STRAIN: J1

SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGATCGCAGC GAGGCTGCCC GCCGGTAACC CCGATAGGTC CACACCACGT 50  
ATCCGGCCGG TTACACCTTC TCGACAGGGG CAATCGAGAC CGAGCCCGGC 100  
ATCGCGATTA CGGCTACCCT GAAAAGAGCA ATGAACGGGG TGAGCACCAG 150  
GTAGGTCGAT GAACACTTTC TTCTCCTCAG ACCAGGTCTC GGCGCCCGAT 200  
CGCGTCGCGC TCTGGCACGA TGTCATCTGC CGTAGCTATG TCCCGCTCAA 250  
CATCACCTTC ACGAGCGAGC AACCCTTCAT CGGTACGGTC TCGACGGGCA 300  
ACTTGGGCAC GGTACGTATC GCGACGTCCT CGTCACTGCC CCAACAGATC 350  
ACCCGCACTC GTCGCTTGAT CAGGCAGGAC GAGCGTGAGT ACCTCATGGT 400  
TGGGGTGCAG TCCGCCGGCC ATGCACTCGT GCAGCAGCAC GGCAGAACTG 450  
CACGAGTCGG TCGCGGTGGA CTGGTCTTCT GGGACACCCG CCATCCCTAC 500  
GACATCCTCT TCCCGACAGA CTGGAGGATG AGCGTATTCC AGTTCCCGCG 550  
ATACTCTTTC GGCTTCACCG AAGACTTCAT CGGCAGGATG ACCGCGGTGA 600  
ACGTCGGGGG CGATCGCGGT ATCGGCCGAG TGGTTTCATC CTTTCATGACA 650  
AGCATCAACG ATGCGACCGA CGCAGGAGAC TTGGCGGAGG TAGCTTCACT 700  
CCACAACAGT GCTGTGATC TTCTGTCAGC GGCGATACGG ACCGAGCTTG 750

CCGATCAAGC CGCCGCCTCC GACGGCCTAC TCGAGTGTGT GCTCGCGTAT 800  
 ATCCGACAGA ACCTGGCCGA CCCGAACCTG TGTGCCTCAC AGATCGCGGC 850  
 5 GGAACACAAC GTCTCTGTGC GGACCCCTCCA CCGACTGTTC TCGGCCACGG 900  
 GACAGGGCGT GGCCGAACAC ATCCGTAACC TCCGACTCGA GCGCATCAAG 950  
 10 ACTGAGCTGG CAGACCCAAC GAGCCGGCGA TATACGATCA GCGCTTTGGC 1000  
 GAGAAAATGG GGGTTCCTCG ATCCCTCAAC GTTCTCACGC GCGTTCAAAG 1050  
 ACGCCTACGG CATCACTGCC CGAGAGTGGG CGGCTTCTGC ATCAGCATCA 1100  
 CCGACGGAGG TTTCGTAGGA AGAGCCCGGT CTCCGGCCTG CCCTTGTTCTG 1150  
 CTTGCGCACC GTTCGGTCCG TCGCTTCCGA TGAAGCCGGA GCCGGCAGGT 1200  
 20 TGGCTTCCTC CCGCGATCCG ATCGCTCGGG GATTGTCCGG GGCACCGCTG 1250  
 GTGACCTCCA GTGCTGCTCC GGCCTGGTGT CCGCGATCGG TGTGCCCCCTG 1300  
 CCCCgATGCA TCCGGGCCGT GATGCCAGTG CTCGGCAGGA CCCACCGGCG 1350  
 25 ACGACGGCCA GCATGACCCA TGGACCGGTC GGTCGAATTC 1390

### Claims

1. A regulatory factor substantially containing an amino acid sequence represented by SEQ ID No:1 and capable of activating a nitrilase gene promoter.
2. A regulatory factor according to claim 1 whose activating activity is enhanced by the presence of a nitrile.
3. A regulatory factor according to claim 2 wherein the nitrile is isovaleronitrile.
4. A regulatory factor according to any one of claims 1 to 3 in substantially isolated form.
5. A regulatory factor gene containing DNA coding substantially for an amino acid sequence represented by SEQ ID No:1.
6. A regulatory factor gene according to claim 5 wherein the DNA coding substantially for an amino acid sequence represented by SEQ ID No:1 is represented by SEQ ID No:2.
7. A regulatory factor gene according to claim 5 or 6 in substantially isolated form.
8. A recombinant plasmid containing the regulatory factor gene of claim 5 or 6, a nitrilase gene containing a promoter region, and a DNA region capable of replicating in a microorganism belonging to the genus *Rhodococcus*.
9. A recombinant plasmid according to claim 8 wherein the DNA region capable of replicating in a microorganism belonging to the genus *Rhodococcus* is a member selected from a group consisting of plasmids pRC001, pRC002, pRC003 and pRC004.
10. A transformant transformed with the recombinant plasmid of claim 8 or 9.

11. A method of preparing an organic acid from its corresponding nitrile which comprises (a) bringing the acid into contact with a culture of a transformant according to claim 10 under conditions in which the transformant express nitrilase and in which said nitrilase is able to convert the acid to its nitrile; and (b) recovering the nitrile.

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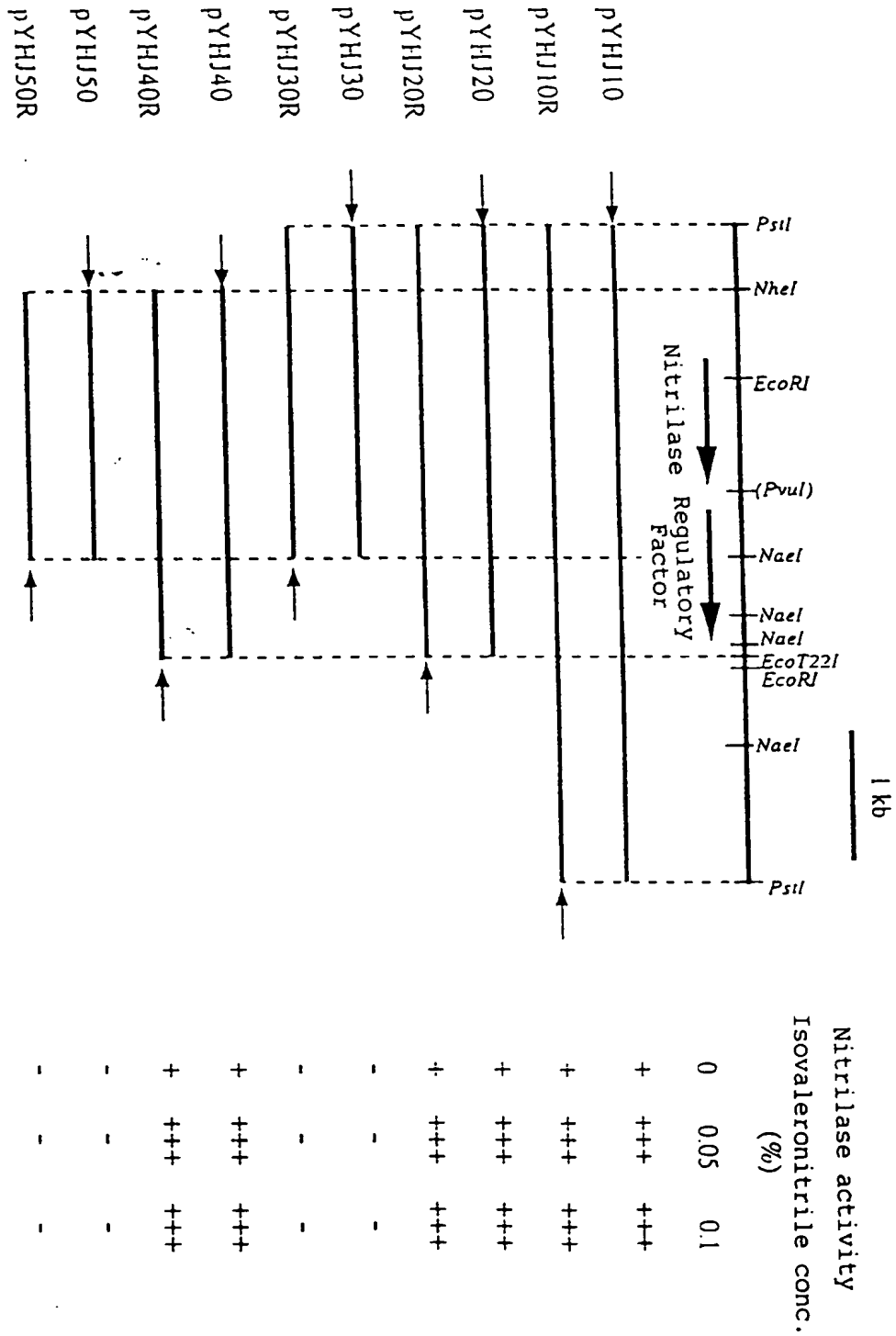
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FIG. 1





(19)



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(54) **A regulatory factor involved in expression of nitrilase gene, and its gene**

(57) The invention relates to a regulatory factor substantially containing an amino acid sequence represented by SEQ ID NO:1 and having the action of activating a nitrilase gene promoter, a regulatory factor gene containing DNA coding substantially for said regulatory factor, a recombinant plasmid containing said regulatory factor gene, a nitrilase gene containing a promoter region and a DNA region capable of replicating in a micro-

organism belonging to the genus *Rhodococcus*, and a transformant transformed with said recombinant plasmid.

According to the present invention, there are provided a regulatory factor gene containing DNA coding substantially for a regulatory factor having the action of activating a nitrilase gene promoter, a recombinant plasmid containing said regulatory factor gene, and a transformant transformed with said plasmid.



European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 96 30 5253

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The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 29 October 1997	Examiner Donath, C
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>&amp; : member of the same patent family, corresponding document</p>			

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